Expressional regulation of the ABCG2 transmembrane transporter by alternative promoter usage and the presence of 5' UTR mRNA isoforms

Theses

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Introduction

The aims of this doctorate study included the identification of new transcriptional and translational mechanisms behind the regulation of ABCG2 gene expression, as well as the development and optimization of transgene delivery methods needed for the study.

ABCG2 is a xenobiotic transmembrane transporter often being responsible for the emergence of the multidrug resistance phenotype in cancer cells. Moreover, its mutations have been associated with predisposition to certain diseases (like gout and protoporphyria) and its expression also plays a crucial role in the protection and maintenance of various stem cell populations. These facts highlight the clinical importance of understanding the mechanisms regulating the gene expression of ABCG2. Our former knowledge about ABCG2 mostly included information about the tissue and cell line specific expression pattern of the gene, the role of signaling pathways on its expression resulting from environmental stimuli, and the function of different micro RNAs regulating ABCG2 transcripts. Much less was known about the role of the multiple promoters described in the ABCG2 gene, and whether the variable first exonic sequences transcribed from these alternative promoters serve as regulators of the gene's expression on the post-transcriptional and translational levels. Because these exon 1 variants represent different 5' non-coding (UTR) regions of the mRNA, only few studies have focused on their roles in contrast to the coding region, or the 3'UTR containing important miRNA binding sites. However, the regulatory function of 5' UTR's observed in other genes and the tissue specific distribution of ABCG2 5' UTR variants suggested a potential role in fine tuning the expression of ABCG2.

Based on these the aims of our study were the following:

1. Unify the nomenclature of already described 5' UTR variants that can be also found in databases. Validate their sequences and their cell-line specific gene expression before further investigations.
2. Examine the expression pattern of these variants in different cell lines following certain chemotherapeutic drug treatments.
3. Investigate the role and expression pattern of these variants in stem cells either exposed to chemotoxic stress or left untreated.

4. Search for possible mechanisms behind the role of different 5' UTR variants play in post-transcriptional and translational regulation. Such mechanism could be the alteration of mRNA stability, or the usage of alternative translation initiation sites.
5. Develop and optimize a *Sleeping Beauty* transposon based transgenesis system, which could be used later to efficiently deliver the ABCG2 transgene into mammalian genomes, even in the case of stem cell lines. This would greatly improve the investigations aiming to better understand the function and regulation of ABCG2 in the future.

Methods

In the frame of this study we utilized several different molecular and cell biology methods in order to answer our questions and test our hypotheses.

The sequences of the investigated ABCG2 first exon variants were cloned from complementary DNA obtained by reverse transcription of total RNA samples isolated from MCF-7/Flv cells. For this, sense primers specific for each exon sequence were used together with a common antisense primer binding to exon 2. The used primers also contained restriction enzyme recognition sites by which the amplified sequences could be inserted into expression plasmids.

Endogenous expression of the mRNA isoforms was measured in several cell lines: HEK293, HUES1, HUES4, HUES9, MCF-7, MCF-7/Mx, MCF-7/Flv and CHRF. MCF-7/Mx and MCF-7/Flv denote the mitoxantrone or flavopiridol-selected derivatives of the parental MCF-7 cell line. Quantitative measurements of the endogenous expression were performed by reverse transcription quantitative Real-Time PCR (RT-qPCR), for which we had designed TaqMan assays specific for each 5' UTR variant. The specific sense primers and the probes bind to the 3' region of the alternating exon 1 sequences, thus providing the specificity of the assays. Efficiency and possible cross-reaction of the assays were tested by dilution standards of plasmids containing each exon1 sequence.

mRNA stability of the isoforms was also determined by measuring the endogenous expression in the MCF-7/Flv cell line which expressed all four variants. For this, cells were treated with the transcription inhibitor actinomycin D and changes in mRNA quantities were followed for 24 hours using the above mentioned RT-qPCR method.

The effect of the different 5' UTRs on gene expression was measured in an artificial expression system. For this, the expression plasmids mentioned above were transfected into HEK293 cells, which did not exhibit endogenous expression of the ABCG2 protein. The four different plasmids encoded a distinct mRNA isoform, as well as a GFP transgene which was used to determine transfection efficiency, and to serve as a control for quantitative measurements of mRNA and protein levels. In the cell populations transfected with different expression plasmids we measured and compared the quantity of ABCG2 protein on the cell surface, the transporter activity of ABCG2 and the total amount of the protein, as well as the amount of ABCG2 mRNA.

For the quantitative measurement of protein expression, the following methods were used:

1. Immunofluorescence based flow cytometry (FACS) method where we used Alexa 647 conjugated 5D3 antibody to label the ABCG2 protein in the plasma membrane of the cells. Following standard protocols, the cells were treated with the specific inhibitor Ko143 prior to 5D3 labelling to increase the specific detection of ABCG2 (Özvegy-Laczka et al., 2005).
2. Fluorescence based cytometric measurement (FACS) quantifying the mitoxantrone extrusion capacity of ABCG2. This mitoxantrone efflux assay can be used to determine activity and function of ABCG2 in the cells.
3. Western blot to semi-quantitatively determine total amount of the cellular ABCG2 protein. For this, BXP-21 primary antibody was used.

Statistical analyses of our results were performed in R Studio program package applying Student's t-test. The decay curves of mRNA stability measurements were determined using the Origin3.5 software "exponential decay" function.

Results

1. We identified all the main first exonic sequences described previously in human cell lines showing endogenous ABCG2 expression. In concordance with previous results we found the E1B/E1C splice variants, and proved the presence of E1A in MCF-7 cells showing high expression of ABCG2. The expression of E1U was found only in few cell lines. Parallel with the identifications and validation of the first exonic sequences we collected literature data about the previously used names of the variants and created a consensus nomenclature to be used during our investigations.

2. We could reproduce previous findings by showing a highly elevated level of ABCG2 in resistant cell lines selected with mitoxantrone or flavopiridol. This was observed not only for the total ABCG2 mRNA population but for the E1B, E1C and E1A containing mRNAs, all of which had been found in parental MCF-7 cells as well. However, the E1U isoform was detected only in MCF-7/Flv cells which suggested that the two drugs had different effects on the cells, activating different responsive signaling pathways and gene expression patterns.

3. We investigated the expression pattern of the isoforms in human embryonic stem cell lines, namely in HUES1, HUES4 and HUES9. In concordance with previous findings we could show that the E1A isoform was expressed in a relatively high level compared to the constitutive E1B/E1C isoforms, while E1U was not detected in stem cells. Following this we investigated the time-dependent changes in ABCG2 expression in HUES9 cells either exposed to chemotoxic stress or left untreated for 3 days. It was previously known that stem cells lose their pluripotency during differentiation. We also observed this phenomenon, both in the case of rapid differentiation induced by mitoxantrone exposure and in the case of slower, spontaneous differentiation of untreated control cells. However, a main difference was found between the two populations: in control cells, the E1A isoform retained its expression while the levels of E1B/E1C were dropped dramatically, whereas in mitoxantrone treated cells, the levels of all variants were reduced to a similar extent.

4. In artificial expression systems, where we investigated the four different mRNA variants separately, we found a notable difference between cells expressing the E1U or

the other isoforms. In the case of E1U isoform, the detected amount of surface ABCG2 was 60% lower and the amount of ABCG2 mRNA was 30% lower than in the case of other isoforms, and we also found a significant decrease in our western blot measurements. No significant difference was observed between the other variants.

5. As our findings indicated different behavior of isoforms E1A and E1U in certain cell types and under certain circumstances, we investigated whether they contained special regulatory elements that could explain the functional relevance of these variants. Using *in silico* methods we identified a potential upstream, in frame positioned translational initiation GTG codon in variant E1A, and found an upstream open reading frame (uORF) in variant E1U which began with an ATG codon. As both elements could potentially influence translational activity, we tested their functionality: we found no activity of the alternative GTG initiation codon whereas we could find a very weak activity of the uORF in variant E1U.

6. We established a *Sleeping Beauty* based transposon gene delivery system and tested it in HEK293, HeLa and HUES9 cells. The system included plasmids containing the transgene in a transposon cassette and plasmids encoding either the SB100x, the SB32 or the SB11 hyperactive transposases.

Conclusions

In the frame of the current study we investigated the expression pattern and efficiency of the four main 5' UTR mRNA isoforms of ABCG2 in different cell lines and under different circumstances. In order to do this, we first had to validate that the sequences cloned and investigated in our studies corresponded to the exon sequences investigated and described previously (Nakanishi et al. 2006; Apáti et al. 2008; Campbell et al. 2011), moreover, we also unified the names of these sequences as they had not been used consistently. Our results were in agreement with the previous findings that the expression of ABCG2 5' mRNA isoforms show typical, tissue and cell line specific distribution (Nakanishi et al. 2006; Apáti et al. 2008; Campbell et al. 2011). While the presence of the first described variants E1B/E1C can be considered as constitutive in ABCG2 expressing cells (Nakanishi et al. 2006; Apáti et al. 2008; Campbell et al. 2011), the E1A variant can usually be found in cells showing an elevated ABCG2 expression, like in certain cancer derived cell lines (Nakanishi et al. 2006; Apáti et al. 2008; Campbell et al. 2011), in stem cells (Apáti et al. 2008) and in placenta (Campbell et al. 2011). The E1U variant seemed unique to megakaryocytic cell lines (like CHRF) and dendritic cells (Apáti et al. 2008) and it is hypothesized to play a functional role in these cell types as in megakaryoblastic leukemia patients, the expression levels were associated with survival rates (Campbell et al. 2011). In addition, we could detect the E1U variant in flavopiridol selected MCF-7 cells as well, providing support for the hypothesis that the regulation of E1U is independent from the other variants. Most likely it is regulated by different pathways and might provide a protective role in chemotoxic stress resistance, and therefore selective advantage for the cells under certain circumstances. Similar conclusions could be drawn in the case of the E1A isoform, which showed a unique expressional behavior in human embryonic stem cell lines. As the stable expression pattern observed for this variant was similar to the expression pattern of the pluripotency markers Oct4 and Nanog it is tempting to hypothesize a stem cell specific regulation for the E1A promoter. It was observed previously that variants E1C and E1A could respond differently to certain signaling pathways (de Boussac et al., 2012), but this phenomena hadn't been described in stem cells.

Although we did not find significant difference in the mRNA stability of the four 5' UTR mRNA isoforms, we measured significantly lower ABCG2 protein and mRNA expression in cells expressing isoform E1U relative to cells expressing other variants. This observation supported the hypothesis that the presence of alternative first exons in the ABCG2 mRNA population is not simply a consequence of different promoter usage but also has a functional

role in post transcriptional and translational regulation of gene expression. We aimed to investigate the details of this potential regulatory mechanism in the case of E1A and E1U. The upstream, in frame GTG codon present in the former variant could result in a protein variant with different localization and function via an alternative translational initiation and N-terminal sequence (Touriol et al., 2003); however our experimental results did not support this hypothesis. The uORF found in E1U could be a potential cause for the lower translation efficiency observed in E1U containing mRNAs, however the very weak activity of this translational unit suggested that other, yet to be revealed mechanisms could also be responsible for the decreased protein level in the case of E1U containing mRNAs (Campbell et al., 2011). Our experimental results raised new questions regarding the regulatory mechanisms and functional relevance associated with 5' UTR variants of the ABCG2 mRNA. Answering these questions would require methods that introduce new, more efficient transgene delivery methods to create stably expressing cell lines. Such method could be the *Sleeping Beauty* (Ivics et al. 1997) based transposon system which we tested and optimized in several cell types, including human embryonic stem cell lines.

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